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We compared O_2 delivery in isolated hearts (1) by red blood cells (RBC) as a function of oxygen dissociation curve position (P_{50}) and (2) by purified hemoglobin in direct comparison to RBC. Hearts were retrograde perfused and performed isometric work by compressing a left ventricular fluid-filled balloon. A right shift of the oxygen dissociation curve (due to incorporation of inositol hexaphosphate into red cells) improved function and myocardial oxygen consumption. Hemoglobin, at equivalent hemoglobin concentration and P_{50}, was indistinguishable from RBC in this model. These findings have important relevance to the Army's blood program.

We also studied the effect of a new allosteric modifier of hemoglobin function (termed RSR-13), which powerfully shifts the oxygen dissociation curve to the right, on cardiac output and its distribution in lightly anesthetized rats. We found no effect on either cardiac output or on absolute or relative blood flow to any organ. This finding is quite surprising finding and runs counter to standard physiologic dogma and most previous work. As such it has potentially important implications for basic physiologic concepts but requires further study.

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CONTENTS

CONTENTS (continued)

INTRODUCTION

In clinical situations when large volume transfusions of red blood cells are necessary, the ability of the erythrocytes to unload oxygen is believed to be of considerable importance. This is because erythrocytes stored under standard blood bank conditions have decreased 2,3-diphosphoglycerate (DPG) content leading to increased oxygen affinity and potentially decreased oxygen delivery. Tissue oxygenation could thus be impaired after transfusion of stored red blood cells, especially in situations when other conditions, such as reduced blood flow or hypothermia during cardiac surgery, are present. This issue is of special concern to the military, as massive transfusions are often necessary for treatment of wounded persons, and the available blood at forward locations will always have been collected some weeks earlier. In addition, human and animal studies have provided some evidence that transfusion of DPG-enriched erythrocytes may improve oxygen delivery over and above that possible with normal erythrocytes. A major undertaking of this project was therefore to clarify and quantitate the importance of blood oxygen affinity in oxygen delivery. If found to be of importance, this would open up new therapeutic possibilities during resuscitation from injury and in treatment of a broad range of other conditions having insufficient oxygen delivery.

A second part of this project dealt with hemoglobin as a blood substitute. Purified hemoglobin preparations have been under development as alternative oxygen carriers for many years, but their use has been hindered by a number of problems including toxicity, high oxygen affinity and short intravascular half life. Recent developments in purification methodology and use of various techniques to increase intravascular persistence and P_{50} have yielded products of considerable interest and potential clinical utility. Despite this progress, there have been relatively few rigorous studies allowing quantitative conclusions about how they function in oxygen delivery. This is of great importance in view of their many major differences from red cells.

BODY

The body of this report is divided into three major sections. For the convenience of readers, the tables, figures and references pertaining to each section are included with that section. The following is a brief summary of each section. (Each of these sections is being prepared for submission as an original article.)

Section I provides direct evidence that decreasing blood oxygen affinity by incorporating inositol hexaphosphate into red cells enhances oxygen delivery to the isolated heart and improves its function. Noteworthy was the finding that a rightward shift was able to produce partial reversal of ischemic effects during low flow ischemia. The conclusion is that the oxygen affinity of blood is indeed physiologically important and that its manipulation may provide an important tool for treatment of casualties of warfare and persons with a variety of ischemic and hypoxic disorders in civilian medicine.

Section II provides data directly comparing efficacy of red cells and hemoglobin in terms of oxygen delivery. The somewhat surprising finding is that they are of very nearly equal efficacy in the model employed.

Section III provides data on cardiac output and its distribution after infusion of RSR-13. This new drug, which shifts the oxygen dissociation curve to the right, was synthesized during the early years of this project and was made available by its developers for physiologic study. This marks the first clinically feasible way of shifting the oxygen dissociation curve. Because of its enormous relevance to this project, we are pleased to include early results. To our great surprise, rats given RSR-13 do not manifest changes in total or regional blood flow, responses which are observed when blood oxygen affinity is altered by other means. While a number of hypotheses are explored as to why this may be so, additional studies at the microvascular level will be required to explain why this is so.

Section I

LOW O_2 AFFINITY ERYTHROCYTES

IMPROVE CARDIAC PERFORMANCE

Effects of Perfusion Rate and P_{50} on O_2 Delivery To Isometrically Working Heart

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Running title: Cardiac performance: P_{50} and flow rate

Key words: blood oxygen affinity, oxygen dissociation curve, P_{50}, inositol hexaphosphate, IHP, isolated heart, rabbit

INTRODUCTION

In clinical situations when large volume transfusions of red blood cells are necessary, the ability of the erythrocytes to unload oxygen is believed to be of considerable importance. Erythrocytes stored under standard blood bank conditions have decreased 2,3-diphosphoglycerate (DPG) content leading to increased oxygen affinity and potentially decreased oxygen delivery. Tissue oxygenation could thus be impaired after transfusion of stored red blood cells, especially in situations when other conditions, such as reduced blood flow or hypothermia during cardiac surgery, are present. Human studies have shown that transfusion of DPG-enriched erythrocytes may improve myocardial performance. Unfortunately the rejuvenation technique used to increase the DPG content of the cells produces only a limited and short-lived decrease of oxygen affinity.

In recent years there have been reports on several methods to shift the blood oxygen dissociation curve to the right, i.e. decreasing the oxygen affinity. One method depends on incorporation of inositol hexaphosphate (IHP) into erythrocytes. As the RBC is normally impermeable to IHP, this requires that RBCs be lysed and resealed. IHP has a strong affinity for the DPG-binding site of the hemoglobin molecule and acts to decrease oxygen affinity of hemoglobin. Animal studies have shown that transfusion of IHP-loaded erythrocytes with low oxygen affinity may play a role in the protection of ischemic tissues.

The isolated heart model of Langendorff has been extensively used to study the effects of various perfusion fluids. The model offers several advantages such as avoidance of autonomic and hormonal effects and ability to monitor blood flow rate, vascular resistance and myocardial performance. We have used an isovolumic rabbit heart model to evaluate myocardial function and oxygen consumption during perfusion with stored erythrocytes (high oxygen affinity) and IHP-loaded cells (low oxygen affinity) at a normal flow rate and during ischemia.

MATERIALS AND METHODS

Preparation of erythrocytes

Krebs-Henseleit (K-H) buffer. The buffer was prepared as follows. The basic solution (118 mM NaCl, 4.7 mM KCl, 2.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.52 mM Na_2EDTA, 25 mM $NaHCO_3$, 11 mM dextrose and 1000U sodium heparin/L) was equilibrated by bubbling with 95% O_2/5% CO_2 at room temperature. Bovine albumin (1.5%) was then added and the solution was filtered (0.22 u).

High-affinity erythrocytes (control cells). Human erythrocytes stored for 6-14 days in standard $CPDA_1$ were washed three times in an isotonic saline solution (1350 G; 5 min); the supernatant and the buffy coat were carefully removed. The red blood cells (RBC) were then diluted with K-H buffer. At this stage the RBC solution was stored in a refrigerator overnight at a hematocrit of 30-40%. The following day the RBC were further washed twice in saline containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 2 mM glucose. Base excess was corrected to approximately 0 meq/L (pH of 7.4 at PCO_2 40 torr) with addition of $NaHCO_3$. The cells were diluted with K-H buffer to give a hematocrit of 25%. The diluted RBC suspension was passed through a leukocyte removal filter (PALL RC100).

Low-affinity erythrocytes (IHP-loaded cells). Packed RBC units were stored for 6-14 days at 4°C. The cells were washed once in isotonic saline and were then passed through a leukocyte removal filter (PALL RC 100). After two more washes in isotonic saline, IHP was incorporated into the cells by the continuous-flow hypotonic dialysis technique previously described by Teisseire et al. (1985). The method was modified by reducing the flow rate of the erythrocytes through the hemodialyzer (Lundia 1C plate dialyzer) to 10 ml/min and by diluting the IHP solution with 0.15 M NaCl (1:1; vol:vol for the first 5 experiments; 1:1.5 for the following experiments) in order to reduce the degree of P_{50} shift. After resealing, the cells were washed once in isotonic saline, once in hypotonic saline (240 mosmol) to lyse the most fragile cells, and two times in isotonic saline containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 2 mM glucose. The erythrocytes were then diluted with K-H buffer containing albumin (1.5%) and stored in a refrigerator overnight. On the day of perfusion the cells were washed once in isotonic saline, once in hypotonic saline and finally three times in saline with $CaCl_2$, $MgCl_2$ and glucose. Then the cells were diluted with K-H buffer with albumin 1.5% and $NaHCO_3$ to achieve a hematocrit of 25% and pH 7.4. The IHP incorporation resulted in a P_{50} of 25-42 mm Hg (mean shift of 16.0 ± 5.1 mm Hg, range 9-26 mm Hg). Mean recovery of erythrocytes was 61%. Supernatant hemoglobin concentration during perfusion was consistently below 0.1 g/dl, and the concentrations of ionized calcium, sodium, and potassium were within the normal range.

Isolated heart preparation

Male New Zealand rabbits weighing between 1.5 and 2 kg were anesthetized with an 8:1 mixture of ketamine/xylazine administered intramuscularly and then given 1000 U of sodium heparin intravenously (IV). The heart was quickly removed after an IV bolus injection of pentobarbital (25-30 mg/kg). The heart was placed in a heated cabinet, the ascending aorta was immediately cannulated, and retrograde perfusion was started at once with either K-H buffer solution or human erythrocytes suspended in K-H solution. A drain was created in the apex of the left ventricle

(LV) by puncture with an 18-gauge needle. A cannulated, fluid-filled balloon connected to a pressure transducer was placed in the LV via a left atriotomy, and a catheter was inserted into the pulmonary artery was to collect myocardial venous effluent. Aortic pressure was monitored by a pressure transducer connected to a stopcock inserted into the line just above the aortic cannula.

Perfusion setup

A schematic diagram of the perfusion setup is shown in Figure 1. The suspended RBC were brought to physiological blood gas concentration and temperature in a primary circuit. From a continuously stirred reservoir, suspended RBC were pumped at a relatively high rate (about 25 ml/l) through a membrane oxygenator (SciMed Life Systems, Minneapolis, MN) and a transfusion filter (PALL Ultipor) to a second overflow reservoir, from which they returned by gravity to the main reservoir. RBC were then propelled by a second pump at the desired flow rate from the overflow reservoir, which also served as a bubble trap, to the heart cannula. Blood passing through the heart was not recirculated. The reservoirs were water-jacketed to maintain a perfusate temperature close to 37°.

Series A. In this series we used normal stored human erythrocytes ("control cells") to evaluate the reproducibility and sensitivity of the isolated heart model and to study the effects of ischemia on left ventricular physiological parameters. Hearts (n=8) were paced at a rate of 160-180/min (4-8 volts, 10 msec pulse duration). They were initially perfused with K-H buffer by gravity at a constant aortic pressure of around 90 mm Hg. The intraventricular balloon volume was set to produce an end-diastolic pressure of 10 mm Hg. The balloon volume was held constant during the experiment so that developed LV pressure (peak systolic pressure minus peak diastolic pressure, LVS-LVD) and its first derivative (dP/dt) reflected the contractile state of the myocardium. Hearts were allowed to stabilize for about 15 min under these conditions. Hearts that did not generate an LVS pressure of at least 60 mm Hg or whose function declined during the stabilization period were discarded. About 20% of hearts were rejected for these reasons.

Perfusion by pump was then started with oxygenated RBC at a constant flow rate of 9 ml/min. This corresponds to a perfusion rate of 2.1 ± 0.2 ml/min/g ventricular wet weight (mean ± SD), which is within the physiological in vivo range (1.8-3.4 ml/min/g) reported for the rabbit heart. This flow rate produced a mean aortic pressure (AoM) of 95 ± 22 mm Hg. Ischemia was then induced by reducing the flow rate to 3.5 ml/min and then to 2 ml/min for at least 5 min. Hearts were allowed to recover for at least 5 min at a flow rate of 9 ml/min after each level of ischemia. Finally flow was interrupted completely for 2 min (total ischemia), after which

the flow rate was returned to 9 ml/min.

Heart rate, left ventricular and aortic pressures were recorded continuously (Gould 481 strip chart recorder). Duplicate arterial (oxygenated blood in the reservoir) and venous (pulmonary artery catheter) blood samples were taken after about 5 min at each flow rate for measurement of pH, PO_2, PCO_2 (Radiometer ABL 30, Copenhagen, Denmark), O_2 content and saturation, and hemoglobin (Hb) concentration (CO-Oximeter, model 282, Instrumentation Laboratory, Lexington, MA). Oxygen dissociation curves (ODC) were determined with either a Hemox Analyzer (TCS Medical Products) or with a Hem-O-Scan (Aminco).

Series B. In this series, hearts (n = 12) were perfused with suspended control RBC immediately after isolation at a flow rate of 9 ml/min, paced (130-180/min) and allowed to stabilize for about 15 min. Each heart was then perfused with control (high-affinity) and with IHP-loaded (low-affinity) RBC at flow rates of 9.0 ml/min, 3.5 ml/min and back to 9.0 ml/min. Duplicate arterial and venous samples were obtained after at least 5 min perfusion. The order of perfusion with control and IHP-loaded RBC was randomly varied such that the order for half of the hearts was C9-IHP9-IHP3.5-C3.5-C9-IHP9, whereas the order for the other half was IHP9-C9-C3.5-IHP3.5-IHP9-C9, where C indicates perfusion with control cells, IHP indicates perfusion with IHP-loaded cells, and 9 and 3.5 indicate perfusion at 9 ml/min and 3.5 ml/min, respectively. In most experiments (n=10) hearts were then exposed to total ischemia (no perfusion) for two minutes once (n=7) or twice (n=3), whereafter the flow rate was returned to 9.0 ml/min. Total experimental time including the stabilization period was 60-90 min.

Histology

Three hearts from series A were examined histologically after various periods of perfusion with control RBC. Muscle fiber structure was intact with normal striations and no visible edema at 1.5 h, the maximal time of any experiment. In comparison with an unperfused control, perfused hearts showed minimal, spotty hemorrhage in the LV myocardium with a tendency of more hemorrhage with increasing perfusion time. These hemorrhages involved <5% of the myocardium. A few punctate hemorrhages could be seen grossly. These changes are not surprising in light of absence of platelets and coagulation proteins in the perfusate and compare favorably with what others have observed (personal communication, M. Vogel). By contrast there was considerably more hemorrhage in the right ventricular wall. Because our study dealt only with LV function, we believe this did not affect our conclusions. The behavior and gross appearance of experimental hearts were similar to those of the histologically examined hearts.

Statistics

Duplicate values obtained for each parameter during each perfusion condition were first averaged. Differences in parameters with changes in flow rate at constant P_{50}, and with changes in oxygen affinity at constant flow rate, were examined by paired *t*-test. Differences as a function of P_{50} in series B following total ischemia were examined by unpaired *t*-test.

RESULTS

Series A. Table 1 shows that arterial blood gases were close to the physiological range. Temperature averaged 35.2 ± 1.1 °C. Figure 2 displays the relationship of several functional parameters (developed LV pressure (expressed as LVS-LVD), dP/dt and LV work (defined as heart rate X (LVS-LVD))) as a function of flow rate (n = 9). At a normal flow rate of 9 ml/min (2.1 ± 0.2 ml/min/g ventricular wet weight), mean value ± SD for LVS-LVD averaged 58 ± 6 mm Hg, positive peak dP/dt 5.3 ± 1.6 (mm Hg/sec), LV work ((LVS-LVD)·HR) 9463 ± 1027 (mm Hg·beats/min). When total O_2 transport (TOT) was decreased to simulate ischemia by reducing flow rate to about 40% of the initial value (3.5 ml/min = 0.8 ± 0.1 ml/min/g), to 20% (2 ml/min ± 0.4 ml/min/g), and to 0% (total ischemia), there were progressive decreases in LV function. Thus, LVS-LVD decreased from the starting value by 39%, 53%, and 78% with the three flow decrements, respectively (Figure 2A; $P < 0.001$ for each decrement by paired *t*-test). Positive peak dP/dt decreased by similar amounts (Figure 2B; $P < 0.001$), changes in LV relaxation rate, as judged by negative peak dP/dt, paralleling changes in +dP/dt. Cardiac work decreased by 38%, 52% and 78%, respectively (Figure 2C). Myocardial oxygen extraction increased by 73% and 163% at 40% and 20% of the initial perfusion rate, respectively (Figure 2D; $P < 0.001$), whereas oxygen consumption decreased by 35% and 44% (Figure 2E, $P < 0.001$). Coronary vascular resistance (mean aortic pressure/coronary flow/min) increased by 60% ($P < 0.01$) and 171% ($P < 0.2$), respectively, at 40% and 20% of basal flow.

Hearts recovered completely with restoration of perfusion to the basal level after the two levels of partial ischemia; there were no statistically significant changes post-ischemia in any parameter from basal values. After total ischemia, there was a 16% decrease from starting value in mean LVS-LVD ($p \leq 0.05$) and a 26% decrease in work ($P < 0.02$); other parameters were unchanged.

Series B. Because this model responded to stepwise decreases in TOT with parallel decrements in function, we evaluated the effect on function of ODC shifts in combination with changes in TOT. Table 1 shows that arterial blood gases and temperature in this series in the circuits with control and IHP-loaded cells were virtually identical and close to the physiological range. Although an arterial PO_2 slightly above the physiological level was employed,

saturation of IHP-loaded cells, as expected, averaged 90 ± 2% (mean ± SD). P_{50} averaged 17 ± 1 torr and 33 ± 5 torr, respectively.

When perfused at 9 ml/min with control cells, LVS-LVD averaged 84 ± 21 mm Hg, peak +dP/dt 8.7 ± 2.2 mm Hg/sec, work (double product) 13173 ± 3047 mm Hg·beats/min, mean aortic pressure 83 ± 28 mm Hg, coronary vascular resistance 42 ± 16 mm Hg·min·g/ml, oxygen extraction 3.1 ± 0.9 ml O_2/dl, and oxygen consumption 0.062 ± 0.022 ml/min/g. These values are shown as 100% in Figure 3. When perfused with IHP-loaded cells, there were small but significant increases in LVS-LVD ($P < 0.05$), peak +dP/dt ($P < 0.05$), work ($P < 0.02$), oxygen extraction ($P < 0.01$), and oxygen consumption ($P < 0.01$). There were no significant changes in mean aortic pressure or coronary vascular resistance.

When the flow rate was reduced to 3.5 ml/min, simulating ischemia, there were highly significant ($P < 0.001$) reductions in mean LVS-LVD, +dP/dt, work, aortic pressure, and oxygen consumption, as observed in series A, while oxygen extraction and coronary vascular resistance increased ($P < 0.001$ and 0.05, respectively); this was true for control and for IHP-loaded cells in relation to their respective controls. Importantly, all parameters of function and oxygen delivery improved significantly ($P < 0.005$ or better) during simulated ischemia when perfusion was switched from control to IHP-loaded cells (Figure 3). The increase in work and oxygen consumption were sufficient to restore 20% and 36%, respectively, of the decrements due to this degree of ischemia.

Complete ischemia caused further decreases in functional parameters ($P < 0.025$ or better). Function during complete ischemia was independent of the type of RBC perfusion (control versus IHP-loaded) preceding the period of ischemia ($P > 0.8$). Upon re-perfusion after ischemia, recovery of function and oxygen consumption improved significantly ($P < 0.001$). These parameters were somewhat better when re-perfusion was carried out with IHP-loaded cells, but the differences from re-perfusion with control cells, possibly because of small numbers, did not attain statistical significance.

Figure 4 depicts in vivo oxygen dissociation curves obtained by plotting arterial and venous O_2 saturation and pressure on perfusate samples obtained under the various conditions described above; these curves re-demonstrate the shift measured under in vitro conditions. At 9 ml/min, mean venous O_2 saturation was appreciably lower and PO_2 appreciably higher with the IHP-loaded cells (table 2, Figure 4). This same pattern was observed at 3.5 ml/min. Accordingly, (A-V)O_2 content difference (Figure 3) was significantly greater during perfusion with IHP-loaded RBC under both conditions and accounts for the significantly greater O_2 consumption observed.

Discussion

The blood ODC is one of several interacting variables believed to be important determining O_2 delivery to tissue. This is because the ODC, along with other factors such as microvascular hematocrit and flow, determine intra-erythrocytic PO_2 and thus the gradient for O_2 diffusion from red cells to tissue. Surprisingly, data on the relative contribution of P_{50} to oxygenation of tissue are rather modest.

The present results indicate that a sizable increase in P_{50} improves myocardial O_2 delivery and function in this model during ischemia. In comparative terms, the increase in P_{50} during ischemia was sufficient to reverse about one-third of the reduction in function and oxygen consumption due to ischemia.

A surprising finding was the improved function and oxygen consumption during perfusion with IHP-containing RBCs at basal flow. This result could imply that hearts were somewhat hypoxic under basal conditions. It is possible that our choice of the basal flow rate, which was based on sparse in vivo data, was suboptimal or that the in vitro heart has a higher O_2 consumption than the in vivo heart. Alternatively, it is possible that the liquid-filled intra-ventricular balloon, being relatively unyielding, produced subendocardial ischemia and that the resulting decrement in oxygen availability could be improved by increased O_2 diffusion due to raising RBC P_{50}. Whatever the explanation, it is of note that the ODC shift did improve venous and presumably capillary PO_2, allowing O_2 extraction, O_2 consumption and function to improve.

Myocardial arteriovenous O_2 extraction in this ex vivo model is less than that observed in vivo in larger animals such as swine, dog and man, where it is possible to collect myocardial venous vein blood under physiological conditions. Data on myocardial O_2 extraction in vivo in hearts of small animals are not to our knowledge available; however, measurements of O_2 extraction in ex vivo hearts of these species by other workers also show less O_2 extraction. It is thus unclear whether the blood-perfused ex vivo heart is less physiological or whether smaller hearts, having appreciably higher heart rates and relatively lower diastolic duration, are adapted to extract less O_2 because they require a higher capillary PO2 for adequate oxygenation. This difference between this ex vivo rabbit heart model and the heart of larger animals makes it difficult to predict whether the beneficial effect of high-P_{50} RBC seen in this study will be observed in larger hearts with higher fractional myocardial O_2 extraction.

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Table 1. Arterial blood gas parameters and temperature

	Temperature °C	PaO_2 Torr	SaO_2 %	$PaCO_2$ Torr	pHa	P_{50} Torr
Series A	35.2 ± 1.1	120 ± 22	97 ± 1	31 ± 4	7.45 ± 0.05	---
Series B						
Control	35.8 ± 0.7	130 ± 20	97 ± 1	34 ± 6	7.41 ± 0.06	17 ± 1
IHP-loaded	35.6 ± 0.7	139 ± 9	90 ± 2	34 ± 2	7.41 ± 0.03	33 ± 5

Values are means ± SD

Table 2. Venous PO_2 and O_2 Saturation

	SO_2 %	PO_2 Torr	PCO_2 Torr	pHv
9.0 ml/min				
Control	71 ± 8	36 ± 6	39 ± 6	7.39 ± 0.05
IHP	58 ± 8	53 ± 15	40 ± 3	7.39 ± 0.02
3.5 ml/min				
Control	54 ± 7	29 ± 4	43 ± 7	7.35 ± 0.05
IHP	42 ± 8	35 ± 7	44 ± 5	7.35 ± 0.05

Values are means ± SD.

Legends to Figures

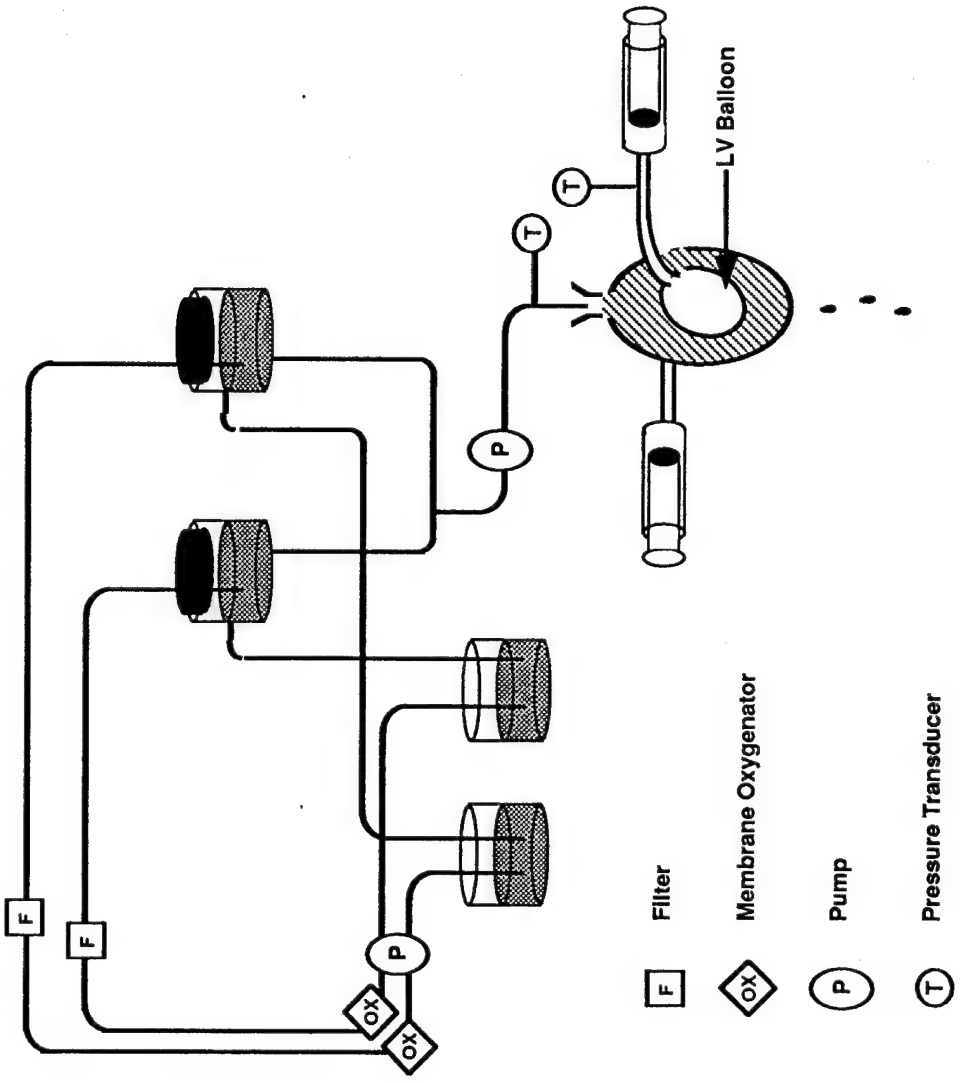
Figure 1. Schematic diagram of perfusion setup.

Figure 2. Effect of various flow rates and of total ischemia on (A) LVS-LVD, (B) peak +dP/dt, (C) LV work, (D) O_2 extraction, (E) O_2 consumption, and (F) coronary vascular resistance. Values of 9.0, 3.5, 2.0, and 0 on X-axis refer to perfusion at respective flow rates of 9.0, 3.5, 2.0 and 0 ml/min.

Figure 3. Interaction of hemoglobin-oxygen affinity and flow rate on (A) left ventricular developed pressure (LVS-LVD), (B) peak +dP/d, (C) LV work, (D) O_2 extraction ($(A-V)O_2$), (E) O_2 consumption (VO_2), and (F) coronary vascular resistance (CVR). C, control RBC perfusion; I, IHP-loaded RBC perfusion. 9.0, 3.5, and 0 on X-axis refer to perfusion at respective flow rates of 9.0, 3.5 and 0 ml/min.

Figure 4. In vivo oxygen dissociation curves during control RBC perfusion and IHP-loaded RBC perfusion. Each point is from a single arterial or venous sample. Upper and lower filled squares represent means for control cells at flow rates of 9.0 and 3.5 ml/min, respectively. Filled triangles represent corresponding means for IHP cells.

Fig 1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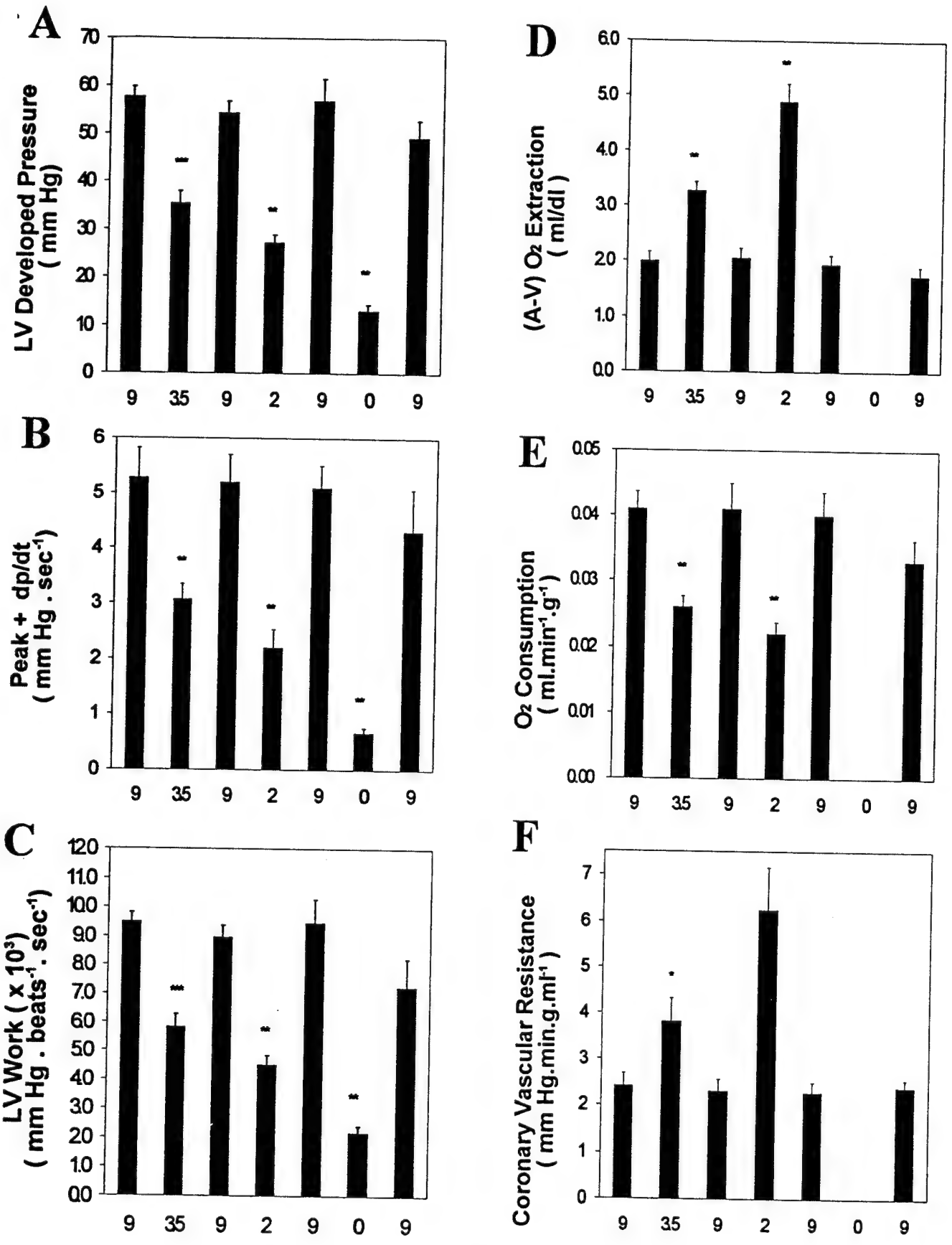
A
LV Developed Pressure (mm Hg)
70
60
50
40
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0
9 35 9 2 9 0 9
D
(A-V) O2 Extraction (ml/dl)
6.0
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B
Peak + dp/dt (mm Hg . sec-1)
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E
O2 Consumption (ml.min-1.g-1)
0.05
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C
LV Work (x 10³) (mm Hg . beats-1 . sec-1)
120
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F
Coronary Vascular Resistance (mm Hg.min.g.ml-1)
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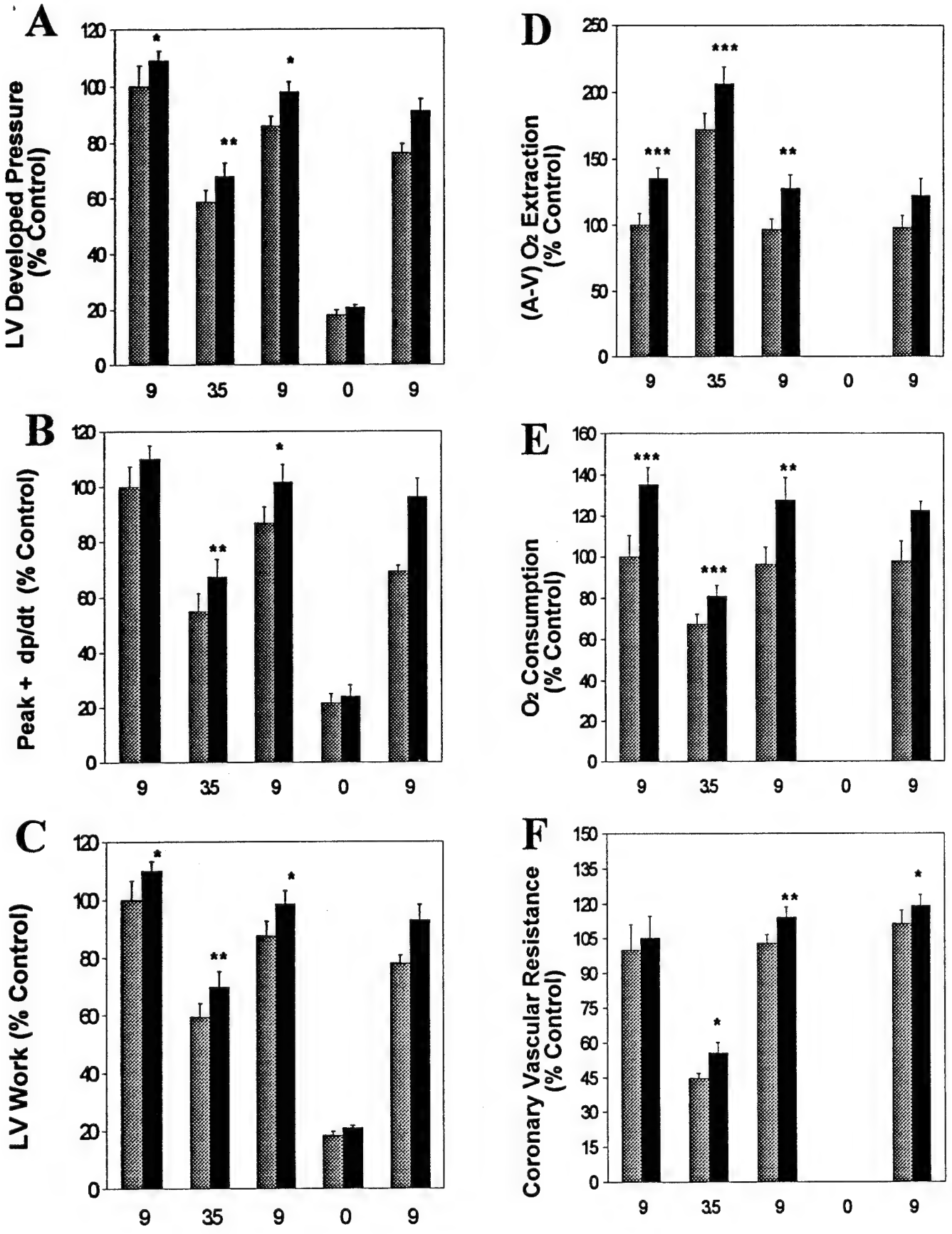
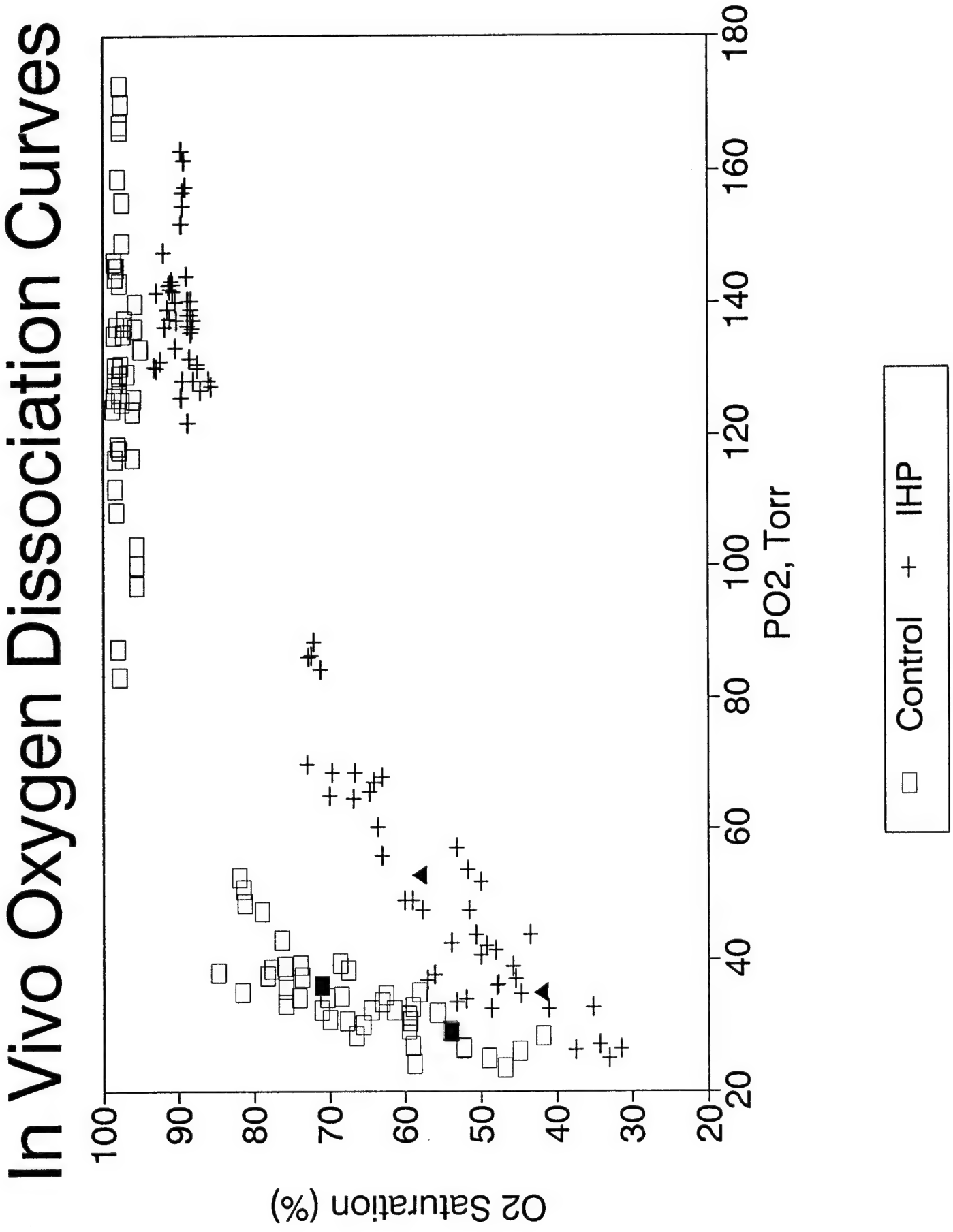
9 35 9 2 9 0 9
Coronary Flow (ml/min)


A
LV Developed Pressure (% Control)
B
Peak + dp/dt (% Control)
C
LV Work (% Control)
D
(A-V) O2 Extraction (% Control)
E
O2 Consumption (% Control)
F
Coronary Vascular Resistance (% Control)
9
35
9
0
9
Coronary Flow (ml/min)


In Vivo Oxygen Dissociation Curves
O2 Saturation (%)
100
90
80
70
60
50
40
30
20
20
40
60
80
100
120
140
160
180
PO2, Torr
Control + IHP

Section II

HEMOGLOBIN SUPPORTS MYOCARDIAL FUNCTION AND O_2 DELIVERY AS WELL AS ERYTHROCYTES

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Running title: Purified hemoglobin and cardiac performance

Key words: Blood substitutes, hemoglobin, isolated heart, rabbit

INTRODUCTION

Purified hemoglobin preparations have been under development as alternative oxygen carriers for many years, but their use has been hindered by a number of problems including toxicity, high oxygen affinity and short intravascular half life. Recent developments in purification methodology and use of various techniques to increase intravascular persistence and P_{50} have yielded products of considerable interest and potential clinical utility. Several of these preparations, for example, have been shown to be superior to crystalloid or colloid, and equivalent to blood, for rescue of animals undergoing major hemorrhage [1-4] and capable of supporting life after virtually complete exchange transfusion [5,6]. Likewise there is good evidence of efficacy in stroke [7,8] and balloon angioplasty models [9].

Perfusion of a tissue bed with a hemoglobin solution differs physiologically in many important ways from perfusion with erythrocytes, some of which might improve oxygenation. First, a hemoglobin solution is less viscous than a suspension of red blood cells (RBC) at equal hemoglobin concentration. Second, there is theoretical [10,11] and experimental [12] evidence from studies of O_2 delivery in skeletal muscle that PO_2 falls markedly in the plasma spaces between RBCs of capillaries, an arrangement that could limit O_2 flux. Since extracellular hemoglobin is distributed uniformly throughout the capillary, more O_2 might be unloaded per unit volume in the case of hemoglobin. Third, hemoglobin, but not RBC, should gain access to collapsed or partially obstructed capillaries; this might be particularly so when flow is sluggish, such as in shock or in re-perfusion injury. Fourth, O_2 diffusion out of the capillary should be improved in the case of extracellular hemoglobin, as experiments employing rapid reaction techniques have demonstrated much faster O_2 release from hemoglobin than from RBC [13]. On the other hand, perfusion of tissues with carefully purified hemoglobin preparations has other complex effects, which could have opposite effects on O_2 delivery. These include increases in blood pressure and vascular resistance due to altered metabolism of nitric oxide, endothelin and probably other mediators, effects which appear to be at least somewhat species, tissue and model specific. These pharmacologic effects are complicated by the possibility of subtle differences among various hemoglobin preparations and by the possibility of toxic effects of hemoglobin [14]. Finally, it must be acknowledged that control of microvascular flow is sufficiently complex that even a through understanding of individual variables would not allow prediction of the final integrated result at the tissue level.

We have therefore studied the ability of hemoglobin, in direct comparison to erythrocytes, to support myocardial function, both at

normal rates of flow and during ischemia. These studies were performed with an ultra-purified preparation of polymerized bovine hemoglobin (PBH). This hemoglobin, unlike human and most other mammalian hemoglobins, does not depend on a cofactor for control of O_2 affinity, retaining a nearly normal P_{50} after separation from erythrocytes. We chose the isolated, isometrically contracting heart model for these studies. This model offers several advantages, including freedom from autonomic reflex changes, capability of measuring blood flow rate and coronary vascular resistance, ability to assess both organ function and O_2 consumption, and the fact that two O_2 carriers can be compared repeatedly under different conditions in the same preparation, thereby improving discriminatory ability.

MATERIALS AND METHODS

Preparation of erythrocytes

Krebs-Henseleit (K-H) buffer. The buffer was prepared as follows. The basic solution (118 mM NaCl, 4.7 mM KCl, 2.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.52 mM Na_2EDTA, 25 mM $NaHCO_3$, 11 mM dextrose and 1000U sodium heparin/L) was equilibrated by bubbling with 95% O_2/5% CO_2 at room temperature. Bovine albumin (1.5%) was then added and the solution was filtered (0.22 u).

Erythrocytes. Human erythrocytes stored for 6-14 days were washed three times in an isotonic saline solution (1350 G; 5 min); the supernatant and the buffy coat were carefully removed. The red blood cells (RBC) were then diluted with K-H buffer. At this stage the RBC solution was stored in a refrigerator overnight at a hematocrit of 30-40%. The following day the RBC were further washed twice in saline containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 2 mM dextrose. Base excess was corrected to approximately 0 meq/L (pH of 7.4 at PCO_2 40 torr) with addition of $NaHCO_3$. The cells were diluted with K-H buffer to give a hematocrit of 25%. The diluted RBC suspension was passed through a leukocyte removal filter (PALL RC100). Pilot studies showed that P_{50} of this preparation was 18-20 Torr.

Hemoglobin preparation. Ultra-purified polymerized bovine hemoglobin (PBH) was obtained from the Biopure Corp. (Boston, MA). This hemoglobin preparation is known to be pyrogen-free and sterile. Its preparation differed slightly from the product prepared by this company for other purposes in that it was dialyzed against Krebs buffer prior to being frozen at -70°C. It was thawed in warm water immediately before use. Concentrations of ionized calcium, sodium, and potassium were within the normal range. Reported P_{50} for this preparation is 20-23 Torr at 37° C.

Isolated heart preparation

Male New Zealand rabbits weighing between 1.5 and 2 kg were anesthetized with an 8:1 mixture of ketamine/xylazine administered intramuscularly and then given 1000U of sodium heparin intravenously (IV). The heart was quickly removed after an IV bolus injection of pentobarbital (25-30 mg/kg). The heart was placed in a heated cabinet, the ascending aorta was immediately cannulated, and retrograde perfusion was started at once with erythrocytes suspended in K-H solution. A drain was created in the apex of the left ventricle (LV) by puncture with an 18-gauge needle. A cannulated, fluid-filled balloon connected to a pressure transducer was placed in the LV via a left atriotomy, and a catheter was inserted into the pulmonary artery to collect the coronary flow. Aortic perfusion pressure was monitored by a pressure transducer connected to a stopcock inserted into the line just above the aortic cannula. Hearts were paced at a rate of 160-180/min (4-8 volts, 10 msec pulse duration). The intraventricular balloon volume was set to produce an end-diastolic pressure of 10 mm Hg. The balloon volume was held constant during the experiment so that developed LV pressure (peak systolic pressure minus peak diastolic pressure, LVS-LVD) and its first derivative (dP/dt) reflected the contractile state of the myocardium. Hearts were allowed to stabilize for about 15 min under these conditions. Hearts that did not generate an LVS pressure of at least 60 mm Hg or whose function declined during the stabilizing period were discarded. About 20% of hearts were rejected for these reasons.

Perfusion setup

Separate parallel circuits were provided for RBC and PBH. Suspended RBC and hemoglobin were brought to physiological blood gas concentration and temperature in a primary circuit. From continuously stirred reservoirs, the RBC suspension and hemoglobin solution were pumped at ~25 ml/l through a membrane oxygenator (SciMed Life Systems, Minneapolis, MN) and a transfusion filter (PALL Ultipor) to a second overflow reservoir, from which each returned by gravity to the main reservoir. The suspended RBC or hemoglobin solution was then drawn by a second pump at the desired flow rate from the overflow reservoir, which also served as a bubble trap, to the heart cannula. RBC or hemoglobin passing through the heart was not recirculated. The reservoirs were water-jacketed to maintain a perfusate temperature close to 37°C. In order to minimize methemoglobin formation in the hemoglobin solution, hearts were first stabilized on the suspended RBC. At this point hemoglobin was rapidly thawed in warm water, brought to physiological blood gas concentration and temperature in its circuit, and corrected to zero base excess. Because some methemoglobin (MHb) was present in the PBH perfusate, total hemoglobin concentration in this perfusate was set slightly higher

than in the RBC perfusate in order to equalize O_2 content in both perfusates at the start of the experiment. The MHb level increased 4-5% during the study.

Hearts were initially perfused at a rate of 9 ml/min. This corresponds to a perfusion rate of 1.9 ± 0.6 ml/min/g ventricular wet weight (mean ± SD), which is within the physiological in vivo range reported for the rabbit heart. This flow rate produced a mean aortic pressure (AoM) of 117 ± 23 mmHg. Each heart was then perfused with RBC and with PBH at flow rates of 9.0 ml/min, 3.5 ml/min and back to 9.0 ml/min. Duplicate arterial and venous samples were obtained after at least 5 min perfusion. The order of perfusion with RBC and PBH was randomly varied such that the order for half of the hearts was C9-H9-H3.5-C3.5-C9-H9, whereas the order for the other half was H9-C9-C3.5-H3.5-H9-C9, where C indicates perfusion with RBC, H indicates perfusion with PBH, and 9 and 3.5 indicate perfusion at 9 ml/min and 3.5 ml/min, respectively. Hearts were then subjected to 2 min of total ischemia and re-perfused at 9 ml/min.

Heart rate, left ventricular and aortic pressures were recorded continuously (Gould 481 strip chart recorder). Duplicate arterial (oxygenated blood in the reservoir) and venous (pulmonary artery catheter) blood samples were taken under each set of conditions for analysis of pH, PO_2, and PCO_2 (Radiometer ABL 30, Copenhagen, Denmark) and O_2 content, O_2 saturation, hemoglobin (Hb) and MHb concentration (CO-Oximeter, model 282, Instrumentation Laboratory, Lexington, MA).

Statistics

Duplicate values obtained for each parameter during each perfusion condition were first averaged. In all cases each heart served as its own control. Differences in parameters with changes of perfusate type and flow rate were examined by paired *t*-test.

RESULTS

Table 1 shows that arterial blood gases and temperature were close to the physiological range and well matched. Starting MHb level in the PBH perfusate was 13.1 ± 0.9%. Arterial O_2 content was well matched, averaging 10.6 ± 1.1 and 10.7 ± 1.2 in the RBC and PBH perfusates, respectively.

Figures 1A-F display changes in several parameters of cardiac function and O_2 transport during perfusion with RBC versus PBH as a function of flow rate (n = 10): developed LV pressure (expressed as LVS-LVD), peak positive dP/dt, LV work (heart rate X (LVS-LVD)), oxygen extraction (A-V O_2), oxygen consumption, and coronary vascular resistance. When perfused with suspended RBC at the basal

flow rate of 9 ml/min (2.1 ± 0.2 ml/min/g ventricular wet weight), mean value ± SD for LVS-LVD was 56 ± 11 mm Hg, positive peak dP/dt 5.4 ± 1.8 mm Hg/sec, LV work (double product) 7566 ± 1880 mm Hg·beats/min, oxygen extraction 2.1 ± 1.1 ml O_2/dl, O_2 consumption 0.040 ± 0.033 ml/min/g and coronary vascular resistance was 66 ± 19 mmHg·min·g/ml. These basal values are shown as 100% in the figures. When perfused with hemoglobin at the same flow rate (second bar in each diagram), there were either slight (statistically insignificant) increases or no change in developed LV pressure, peak +dP/dt, work, oxygen extraction, and oxygen consumption. There was a slight but insignificant decrease in coronary vascular resistance with hemoglobin.

When the flow rate was reduced to 3.5 ml/min, simulating ischemia, there were highly significant reductions in mean LVS-LVD, +dP/dt, work, and oxygen consumption, while oxygen extraction and coronary vascular resistance increased ($P < 0.01$ or better); this was true for RBC and PBH perfusion. Thus VO_2 was limited at this point by rate of O_2 flow. Under these conditions, perfusion with PBH, as opposed to RBC, produced no change or an insignificant improvement in cardiac function and oxygen delivery.

Total ischemia produced a sharp drop in developed LV pressure, +dp/dt and work, all of which returned to baseline levels afterwards. Cardiac function during ischemia and post-ischemic function did not differ between perfusion with RBC and PBH.

A parallel series of investigations were carried in an additional 6 hearts. These differed from the above only in that the starting MHb level was appreciably higher, averaging 22.2 ± 2.9%. Results in this latter series were the same as in the above series with lower starting MHb level, again demonstrating equal or insignificantly improved function and O_2 consumption with PBH (data not shown).

Because the ODC is important to O2 release, and since a decrease in P_{50} of the hemoglobin solution due to methemoglobin could reduce or abrogate any advantage in O_2 delivery of free hemoglobin over red cells, we estimated the position of the in vivo oxygen dissociation curve in post-capillary blood. Figure 2 shows results curves obtained by plotting arterial and venous PO2 and O_2 saturation of *functional hemoglobin*. Analysis discloses no difference in curve position. This was also true after correction of all points to the standard pH of 7.4 (data not shown). Mean values for arterial and venous PO_2 and O_2 saturation at the flow rates of 9 ml/min and 3.5 ml/min are shown in table 2. While SvO_2, when expressed in relation to total hemoglobin, is lower for PBH, there is no difference in SvO_2 expressed in relation to functional hemoglobin or in CvO_2.

DISCUSSION

Purified hemoglobin has been studied in a variety of in vitro and in vivo settings. In nearly all of these studies, investigators have attempted to assess the effect of hemoglobin on organ function and/or oxygen delivery in the presence of RBC. Although there is little doubt from these studies that hemoglobin contributes to tissue oxygenation, this design makes it difficult to determine the exact contribution of hemoglobin; furthermore, any preferential O_2 delivery from either hemoglobin or RBCs would not be identifiable in measurements relying on venous O_2 measurements due to rapid equilibration RBC and hemoglobin in post-capillary blood. The present study, which uses alternating perfusion by hemoglobin and RBCs, is unique in that it allows a direct comparison of hemoglobin with RBC perfusion on hemodynamics and O_2 transport when each of these products serves as the sole O_2 transporter.

These results show that hemoglobin was able to support myocardial function, as judged by pressure generation, dP/dt and work at least as well as RBCs. This was also true for oxygen consumption. This equality was observed both when the coronary perfusion rate was set at the normal level and at a suboptimal level to simulate ischemia. Although we had hypothesized that improved O_2 delivery might be observed with hemoglobin for one or more of the reasons given in the introduction, especially during ischemia, we did not observe convincing evidence for this. Our results with cardiac muscle are thus in general agreement with studies of Biro et al [15] and Hogan et al [16] in isolated canine skeletal muscle, who observed equivalent but not improved O_2 delivery when perfusion by blood and blood plus hemoglobin were compared at equal flow rate and equal total hemoglobin concentration.

There are a number of reasons why we may not have observed improved function. The prediction [10,11] and recent demonstration [12] of low PO_2 in plasma gaps between RBCs of skeletal muscle capillaries may not apply to myocardium, for which comparable data are not available. Likewise, the finding of more rapid deoxygenation of oxyhemoglobin in solution is based on studies by rapid reaction methodology, which may poorly reproduce diffusive conditions in microvessels. The supposed benefit accruing from improved rheology and/or preferential access of hemoglobin solutions to collapsed microvessels may also be an oversimplification of the in vivo setting.

An alternative possibility is that hemoglobin has direct effects which mitigate the expected advantages. Studies of various hemoglobin preparations in whole animals have regularly demonstrated increased systemic blood pressure and vascular resistance, raising concern as to whether vasoconstriction would decrease nutrient vessel perfusion. Indeed, peripheral vascular resistance and vascular resistance of skeletal muscle is known to

increase with hemoglobin, including highly pure hemoglobin [17,18], and could thereby account for the absence of improved O_2 delivery to muscle with hemoglobin as observed by Biro et al [15] and Hogan et al [16]. In the case of myocardium, earlier studies employing isolated rabbit or rat hearts have suggested that hemoglobin solutions also caused vasoconstriction in coronary vessels [19]. However, the degree of vasoreactivity was soon found to vary with the hemoglobin preparation, with some preparations showing marked vascular reactivity at very small doses [19,20]. In general, later studies done with highly pure hemoglobin products displayed reduced or no coronary vasoconstriction [20,21]; in a later study, Vogel et al showed that, with at least one preparation, vasoconstriction occurred at low hemoglobin concentrations but not at physiologic concentration [22]. The lack of an inherent coronary vasoconstrictor effect is especially supported by in vivo studies of Gulati and colleagues [17,18], who found increased coronary flow and decreased vascular resistance in intact animals top-loaded with 400 mg/kg of highly pure unpolymerized disapirin-crosslinked human hemoglobin in comparison to controls, but the opposite in animals given impure hemoglobin [17]. In the present study we also observed no increase in vascular resistance in the heart when perfused exclusively by hemoglobin, at either basal or reduced flow. However, it is not possible in this study, nor in earlier ones, to determine to what extent results are a mixture of vasoconstriction and improved rheology, leaving measured vascular resistance relatively unchanged.

An additional factor believed important in cardiac oxygenation is the position of the oxygen dissociation curve [23,24]. Although in this study the starting P_{50} value of the hemoglobin solution probably exceeded that of stored human RBC by 0-2 mm Hg, a decrease in P_{50} of the hemoglobin solution due to gradual methemoglobin accumulation could lower the P_{50} of the hemoglobin partially abrogate benefit from extra-corpuscular hemoglobin. We therefore estimated the position of the in vivo curve in post-capillary blood by plotting venous saturation and PO_2 measurements at actual pH in cardiac vein blood collected anaerobically from the pulmonary artery. This method has the advantage of permitting an <u>in vivo</u> comparison on the ODC of RBC and hemoglobin, which was valuable given the slow rise in methemoglobin concentration over time. Our results indicate that there is no difference between the two in vivo oxygen dissociation curves under our conditions. This is in accord with our expectation, as the modestly higher P_{50} of the hemoglobin preparation decreased as methemoglobin accumulated; in fact this reduction was almost identical to what one would predict from the classical equation of Roughton [25].

In conclusion, we find that hemoglobin supports the function of this ex vivo heart equal to blood. While certainly reassuring in terms of anticipated uses of hemoglobin in various clinical settings, we did not find evidence of improved O_2 delivery or

function. More exact details of hemoglobin function in O_2 delivery will require studies at the microvascular level.

ACKNOWLEDGMENTS

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Table 1. Arterial blood gas parameters, methemoglobin and temperature

Perfusion Medium	Temperature °C	PaO_2 Torr	SaO_2 %	CaO_2 ml/dl	$PaCO_2$ Torr	pHa	MHb %
RBC	37.2 ± 0.4	140 ± 7	98 ± 0	10.6 ± 1.1	37 ± 3	7.45 ± 0.02	0.2 ± 0.3
PBH	37.1 ± 0.4	153 ± 5	87 ± 1	10.7 ± 1.2	39 ± 2	7.41 ± 0.06	13.1 ± 0.9

Table 2. Venous Blood CvO_2, PO_2 and O_2 Saturation

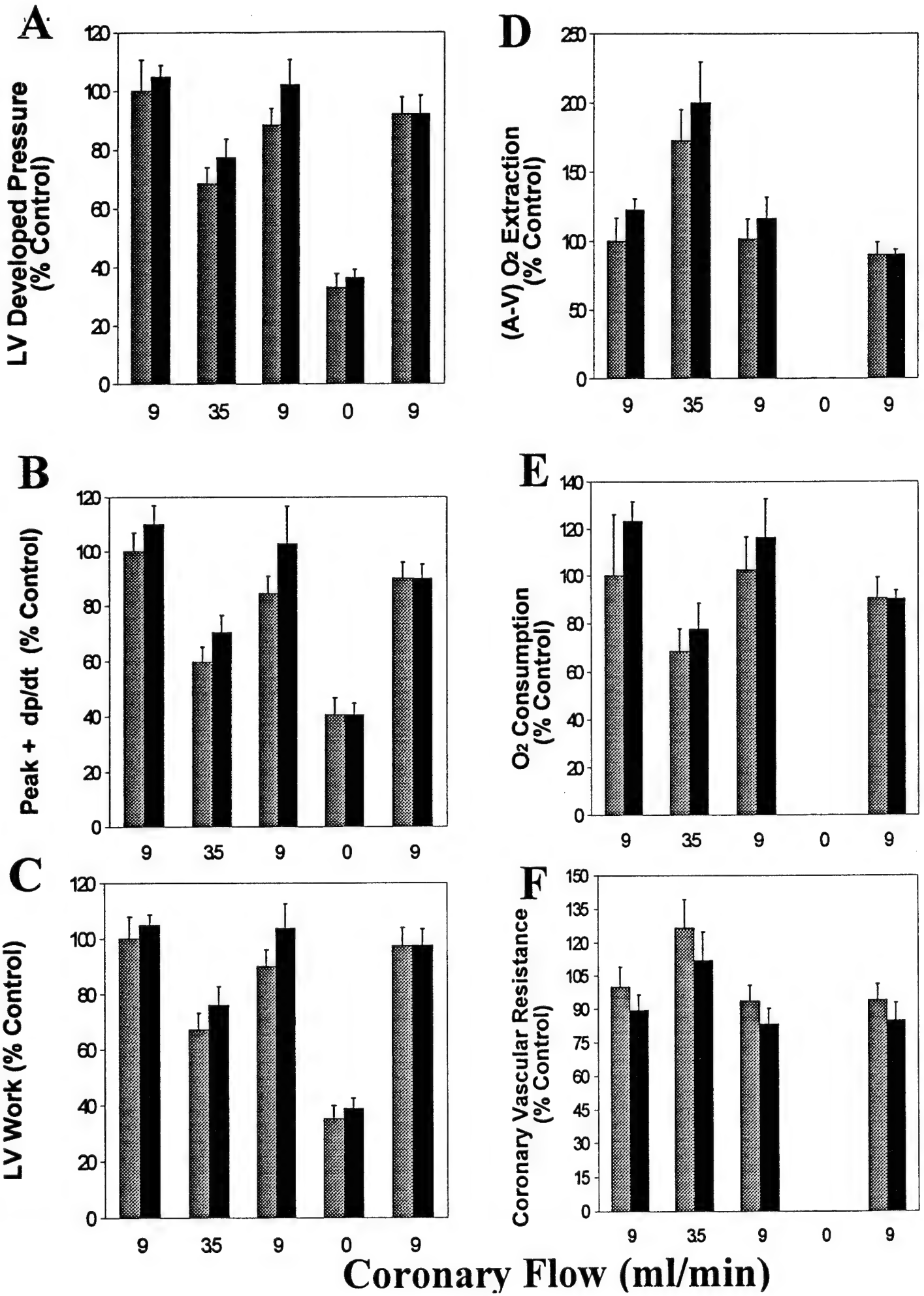
	N	SvO_2[#] %	SvO_2[$] %	CvO_2 ml/dl	PvO_2 Torr	$PvCO_2$ Torr	pHv
9.0 ml/min							
RBC	10	79 ± 10	80 ± 11	8.6 ± 1.2	36 ± 8	41 ± 4	7.43 ± 0.04
PBH	10	69 ± 7	80 ± 8	8.5 ± 1.2	36 ± 7	45 ± 2	7.38 ± 0.09
		$P < 0.05$	NS	NS	NS	NS	NS
3.5 ml/min							
RBC	10	64 ± 13	66 ± 14	7.3 ± 1.6	29 ± 7	43 ± 5	7.41 ± 0.05
PBH	10	58 ± 9	68 ± 11	7.1 ± 1.5	28 ± 7	45 ± 5	7.40 ± 0.10
		$P < 0.05$	NS	NS	NS	NS	NS

[#]Expressed as % total hemoglobin
[$]Expressed as % active hemoglobin

Legends to Figures

Fig 1. Influence of perfusion with RBC versus PBH on LV developed pressure (fig 1A), +dP/dt (fig 1B), LV work (fig 1C), O_2 extraction (fig 1D), and O_2 consumption (fig 1E), and coronary vascular resistance (fig 1F). 9.0, 3.5, and 0 on X-axis refer to perfusion at respective flow rates of 9.0, 3.5 and 0 ml/min; the first bar of each pair at a given flow rate (cross-hatched) is during perfusion with RBC and the second (solid) is with PBH.

Fig 2. Relationship between blood O_2 saturation of functional hemoglobin (hemoglobin available for O_2 binding) and PO_2 during perfusion with red cells (fig 2A) and hemoglobin (fig 2B).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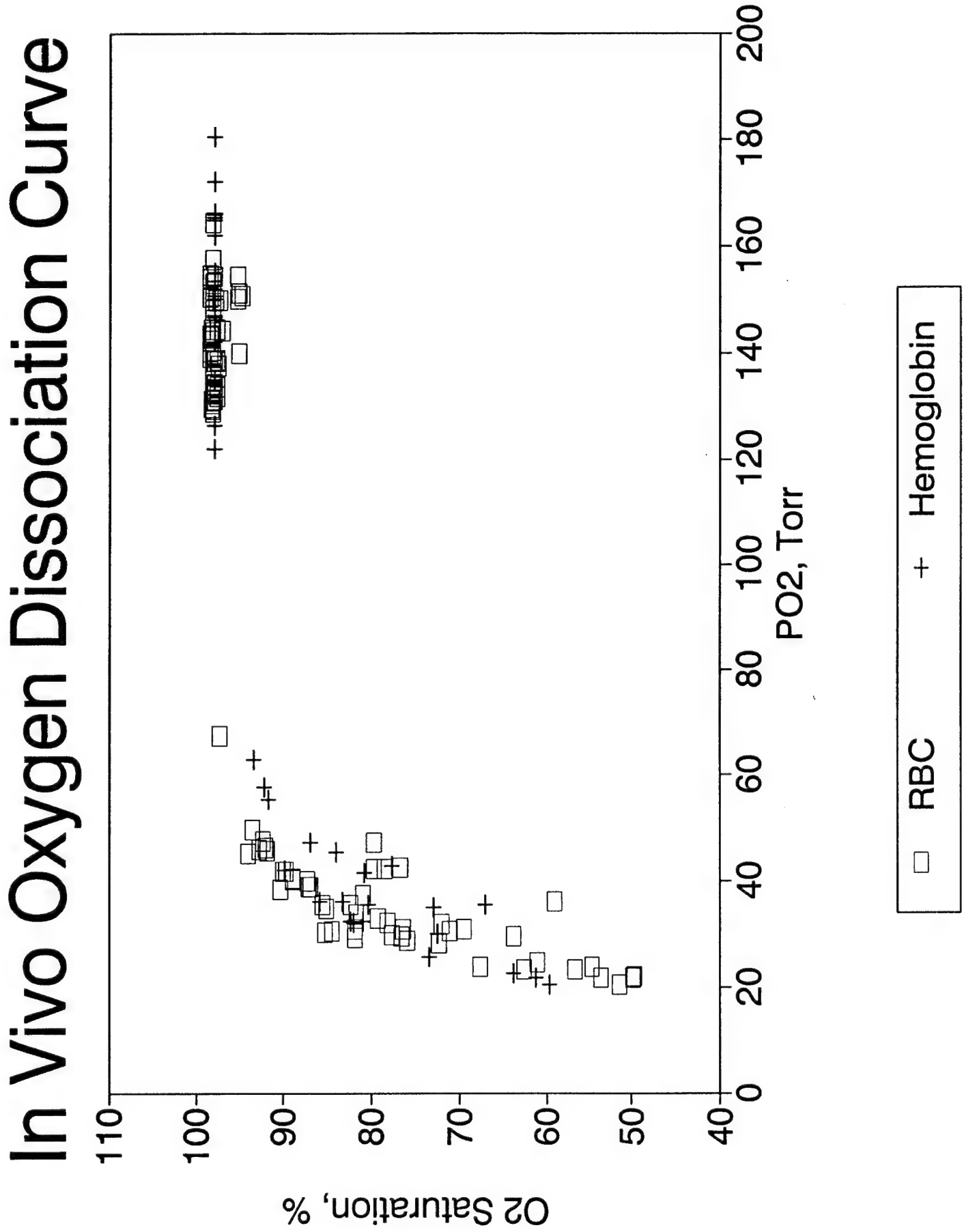
LV Developed Pressure (% Control)
B
Peak + dp/dt (% Control)
C
LV Work (% Control)
D
(A-V) O2 Extraction (% Control)
E
O2 Consumption (% Control)
F
Coronary Vascular Resistance (% Control)
9
35
9
0
9
Coronary Flow (ml/min)

Fig 2


In Vivo Oxygen Dissociation Curve
O2 Saturation, %
110
100
90
80
70
60
50
40
0
20
40
60
80
100
120
140
160
180
200
PO2, Torr
RBC
Hemoglobin

Section III

Effect of Low Blood Oxygen Affinity On Cardiac Output and Distribution

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Running title: Low blood O_2 affinity and blood flow distribution

Key Words: RSR-13, blood oxygen affinity, oxygen dissociation curve, blood flow distribution

Introduction

A number of factors influence the amount of oxygen delivered to the tissues. Total oxygen transport, sometimes termed "offered O_2," is a simple multiple of blood arterial oxygen content and cardiac output. At the microvascular level the amount of oxygen released from blood is equal to that required for tissue oxygen consumption. This movement of O_2 is due to diffusion and is dependent on diffusivity and on the gradient between microvascular PO2 and tissue PO2. The latter is determined in part by the position and shape of the oxygen dissociation curve (ODC).

It is currently unclear how various tissues respond to changes in the ODC. If tissue PO_2 is tightly regulated, a change in the ODC, with the expected changed in PO_2 in microvessels, should trigger a change in local blood flow that would return tissue PO_2 toward normal. If PO_2 is not so regulated in a given tissue, a shift in ODC position should not affect flow except perhaps in the case of an extreme left shift that caused O_2 consumption to fall. Additionally, various tissues may respond differently to the same change in the ODC. For example, Woodson and Auerbach reported that blood flow to brain and heart of rats exchange transfused with high affinity blood rose appreciably, while flow to the major abdominal organs did not change (1). Liard and Kunert studied hemodynamic effects of an acute right shift of the ODC (increased P_{50}) in dogs and in rats, due to exchange transfusion with low affinity blood in dogs (2) and to exchange transfusion with low affinity blood or intravenous infusion of an allosteric effector in rats (3). In those studies cardiac output decreased in parallel with the increase in P_{50}, suggesting a compensatory reduction of blood flow in response to the potential increase in local PO_2. In a separate study they observed a decrease in flow to the rat iliac bed in response to a right shift in the ODC while superior mesenteric flow did not change. On the surface this would seem to imply that different beds respond differently to a change in tissue the ODC and presumably local tissue PO_2.

Abraham and colleagues have recently synthesized a new family of compounds based on the drug bezafibrate, an antilipidemic agent (4). These compounds have the unique characteristic of shifting the blood ODC after injection (5,6). They are known to penetrate the RBC membrane and to bind in the central water cavity of the hemoglobin molecule, remote to the binding site for 2,3-diphosphoglycerate. Of particular interest is RSR-13 ($C_{22}H_{23}NO_4$, a 2-[4-[[9,3,5-disubstituted anilino)carbonyl]methyl]phenoxyl]-2-methylproprionic acid derivative), which shifts the ODC to the right; it is not known to have toxic effects and has undergone limited testing in normal human subjects. We therefore decided to investigate the effect of an acute rightward shift of the ODC due to this agent on cardiac output and on blood flow to various organs as a means to understanding regulation of O_2 pressure in tissue.

Methods

Animal Preparation. Male Sprague-Dawley rats (316 ± 25 g) were anesthetized by intramuscular injection of droperidol-fentanyl (Innovar-Vet, Pitman-Moore), 0.2 $ml \cdot kg^{-1}$. A slightly beveled 15 cm length of PE-50 catheter (Clay Adams, Parsippany, N.J.) was advanced from the right carotid artery into the left ventricle during continuous pressure monitoring. When a stable left ventricular pressure signal was obtained (catheter advanced ~3.5 cm), the catheter was secured in place. Satisfactory catheter placement was confirmed at autopsy. Pilot studies by two color Doppler imaging established that this catheter did not produce valvular insufficiency. Visual inspection also confirmed that the cross sectional area of the catheter small in comparison to the cross-sectional area of the aortic valve. A second PE-50 catheter was placed in the left femoral artery. Both wounds were closed with metallic clips.

Measurement of cardiac output and organ blood flow. Radioactive microspheres, 15 μm, labeled with ^{57}Co and ^{46}Sc, were obtained from Dupont. Stock solutions were prepared by diluting an aliquot of each isotope with a solution of 0.9% NaCl, 10% dextran and 0.1% Tween 80 to a concentration of approximately 300,000 microspheres$\cdot ml^{-1}$. Specific microsphere radioactivity was obtained by counting a known number of microspheres in a gamma counter (Packard model 5780). This was done in triplicate by adhering microspheres to broken glass cover slips, enumerating the microspheres microscopically, and embedding the cover slip fragments in gelatin in a sealed counting tube. These tubes served as counting standards for each experimental run.

After vigorous vortexing and ultrasonic dispersion, shown in pilot studies to effect complete disaggregation, 0.2-0.3 ml of the stock microsphere suspension was transferred to a small stoppered vial and counted. After similar vortexing and ultrasonic dispersion, ~0.2 ml (60,000-130,000 microspheres) were withdrawn and immediately infused into the left ventricle at a rate of 0.01 $ml \cdot sec^{-1}$; this was followed by a 0.4 ml warmed saline flush at 0.02 $ml \cdot sec^{-1}$. A reference blood sample was withdrawn by syringe pump (model 901, Harvard) from the femoral artery at a rate of 1.03 $ml \cdot min^{-1}$. This withdrawal began 10 sec before injection of microspheres and continued for 20 sec after the saline flush. The injection syringe and vial were then counted and the number of microspheres injected determined by difference.

The number of microspheres in each organ was determined by counting the blotted, weighed organs, making appropriate corrections for background and cross counts and dividing by radioactivity per microsphere. Since pilot studies showed that counting efficiency was independent of sample height only to a height of 4 cm, larger

organs were divided among multiple vials and the results summed. An estimate of skeletal muscle microsphere content was based on two samples of approximately equal weight taken from one hind limb (gluteus, hamstrings and gastrocnemius-soleus) and from back (paravertebral musculature). The number of microspheres in the carcass was determined by subtracting from the total number of microspheres injected the sum of microspheres in all counted organs except skeletal muscle. Cardiac output (Q) was calculated according to the relationship

$$Q = R \times T/R$$

where R is the withdrawal rate of the reference sample, T the total number of spheres injected and R the number of spheres in the reference sample. Blood flow to each organ was expressed both as absolute flow and as a percentage of cardiac output.

Preparation of drug. The sodium salt of RSR-13, generously provided by Allos Therapeutics (Denver, CO), was dissolved in 0.45% saline to a final concentration of 109.5 mM (40 mg/ml), which is isotonic. Controls received isotonic saline.

Hemodynamics and blood measurements. Systolic, diastolic and mean arterial pressures and heart rate were measured with a Gould P50 pressure transducer and Gould Brush 481 recorder. Blood gases were measured with an automated blood gas machine (ABL30, Radiometer). Hematocrit was measured by the microhematocrit method. Rectal temperature was measured with a calibrated thermistor probe (model 44TA, Yellow Springs Instrument Co.). Respirations were counted for 20-30 seconds and converted to breaths·min^{-1}.

P_{50} was measured on whole blood samples by a thin film dual-beam spectrophotometric/polarographic method at 37° C (Hemo-Scan, Aminco). This method requires that blood be fully oxygenated and deoxygenated during calibration. Because blood with a large right shift may not be fully oxygenated with the usual oxygenating gas (23.2% O_2/5.1% CO_2/balance N_2), and since use of a higher O_2 concentration in the oxygenating gas would decrease accuracy of the measured ODC, we devised the following procedure. The blood sample was first equilibrated with humidified 95% O_2/5% CO_2/balance N_2 and the balanced spectrophotometer output taken as 100% O_2 saturation. The oxygenating gas was then changed to 23.2% O_2/5.1% CO_2/balance N_2, and this gas and the usual deoxygenating gas (0% O_2/5.1% CO_2/balance N_2) were used and to set the upper and lower PO_2 electrode calibration points and the point for zero O2 saturation. The ODC was then run in the usual way. A separate aliquot of the same blood sample was simultaneously tonometered (model 237, Instrumentation Laboratory) with these latter two gas mixtures, and the pH values measured. The mean of these two pH values was used to correct the measured P_{50} to pH 7.4.

Evaluation of data and statistics. A requirement for validity of this method is homogeneous distribution of microspheres in blood exiting the left ventricle. Hence rats were rejected if the number of microspheres in the left and right kidney differed by more than 10%. One rat was rejected for this reason. All organs contained >400 microspheres with the exception of diaphragm, which usually contained 200-300 microspheres.

Changes in measured parameters within each group from before to after injection of RSR-13 or saline were evaluated by paired *t*-test. An unpaired *t*-test was used to evaluate the significance of between group differences. An unpaired t-test was also used to compare differences between the RSR-13 and saline groups in magnitude of change from before to after injection (i.e., $parameter_{after}$ minus $parameter_{before}$). A probability level of <0.05 was considered significant.

Experimental Protocol

Since the oxygen dissociation curve was right-shifted in rats receiving RSR-13, it was necessary to increase inspired O_2 to obtain adequate O_2 saturation of arterial blood. This was done by placing the rat's upper body in a plastic bag flushed with humidified gas (F_IO_2 40-45%) at a flow rate sufficient to prevent accumulation of CO_2 (1000-1500 ml·min-1).

Animals were studied 60 min after catheter placement (40 min after breathing O_2-enriched air), while still lightly anesthetized. A femoral arterial sample of 300μL was withdrawn for determination of PO_2, PCO_2, pH, and hematocrit, and arterial pressure, heart rate, respiratory rate and temperature were recorded. The first measurement of cardiac output and distribution was then carried out, followed immediately by a repeat measurement of blood gases, hematocrit and vital signs. The exact volume of blood removed to this point (1.8 ml) was replaced over one minute with an equal amount of warmed donor rat blood via the femoral arterial catheter. Next the rat was given either RSR-13 (125 or 150 mg/kg) or an equal volume of saline by intra-ventricular injection over 3 min. Thirty minutes after completion of this infusion the above measurements (arterial blood gases and vital signs, cardiac output and distribution, arterial blood gases and vital signs) were repeated.

After completion of this protocol, a 3 ml blood sample was obtained for P_{50}, and the rat was euthanized with sodium pentobarbital. Correct placement of the LV catheter tip near the apex of the left ventricle was confirmed by autopsy. The endothelial surface was examined meticulously to ensure that subendocardial injection of microspheres had not occurred; in no case was this observed. Blood for basal P_{50} (prior to RSR or saline injection) was not obtained from the experimental rats due to the relatively large volume required and the possibility that this procedure, despite blood

replacement, might confound subsequent measurements. Consequently blood for basal P_{50} determination was obtained in parallel from donor rats and measured concurrently.

Results

Standard P_{50} and blood gases

Basal P_{50} in the experimental group of rats averaged 35.4 ± 5.4 Torr (SD) and increased to 46.3 ± 4.1 Torr after the infusion of RSR-13 ($P<0.001$). Figure 1 shows a representative oxygen dissociation curve from a rat before and after RSR-13.

Arterial blood gas results are summarized in table 1. These are essentially unchanged from before to after each injection and do not differ between the two groups. Although some of the changes are statistically significant, these are very small and not of biological importance. Hematocrit and body temperature also showed no important changes. Based on the measured PaO_2 values in the range of 225-250 Torr and the finding that O_2 saturation at a PO_2 of 140-145 Torr in ODC measurements was ~90%, we estimate that SaO_2 in the RSR-13 group was close to 95%.

Hemodynamics and blood flow distribution

Hemodynamic parameters are shown in table 2. Mean, systolic and diastolic pressure, heart rate and respiratory rate were alike in the two groups at the start of the protocol. Heart rate decreased slightly after injection in both groups, but there was little change in the other measurements. Cardiac output decreased by a similar amount (24% and 22%) in the RSR-13 and control group, respectively, from before to after injection. Peripheral vascular resistance rose significantly in both groups, but the degree of change did not differ between the groups.

Table 3 summarizes values for absolute organ blood flow in response to an infusion of RSR-13 or vehicle. There was no change in flow to heart, brain, kidney, or lungs in either group. Blood flow to the other visceral organs tended to decrease from before to after injection. This was true in both groups for stomach (24 and 37%, respectively) and hepatic portal blood flow (14 and 15%). Spleen and intestinal flow also decreased, though the decrease was not significant for intestine in the control group or for spleen in the RSR-13 group. Flow to diaphragm and to back and leg muscle decreased significantly in both groups, the reductions averaging 29% and 43%, respectively, in the RSR-13 group and 22% and 35% in the control group. Likewise, flow to carcass decreased significantly in both groups (28% and 24%). In none of these instances did the magnitude of change (flow $rate_{after}$ minus flow $rate_{before}$) differ significantly between RSR-13 and control rats.

Table 4 gives corresponding data by organ with results expressed as percentage of cardiac output. Percent of total blood flow to heart and brain increased significantly from the first set of measurements to the second (by 39% and 29% for heart and by 31% and 24% for brain). Percent of blood flow to kidney, intestine, and liver also tended to rise. In no case did the degree of change differ between the RSR-13 and control groups. By contrast, fraction of blood reaching carcass dropped by 16% and 11% in the two groups ($P < 0.001$). This decrease, because of the comparatively large size of the caracas, was approximately equal to the increase in blood flow to other organs. This redistribution of blood flow thus served to keep absolute blood flow to the internal organs relatively constant at the expense of carcass (table 3); however, this redistribution was not influenced by the difference in blood P_{50}.

Discussion

The primary goal of this study was to characterize cardiac output, regional blood flow and distribution of cardiac output in response to an acute right shift of the ODC caused by an allosteric modifier of hemoglobin function, RSR-13. Theoretically, a right shift in the ODC curve should result in an increase in tissue PO_2 due to an increase in PO_2 in the microvasculature. If tissue PO_2 is tightly regulated, this increase in P_{50}, assuming no concurrent change in oxygen consumption, might be expected to cause vasoconstriction in some if not all tissues in order to restore tissue PO_2 to normal. We found no evidence of this occurring in this study.

There are several reasons why this response may not have occurred. One possibility is simply that tissue PO_2 is not tightly regulated. This however seems somewhat counter intuitive, given the well known reciprocal relationship between ODC and hematocrit, mediated by erythropoietin, and the tight coupling between metabolism and blood flow in brain, heart and other metabolically active tissues. A second possibility is that tissue PO_2 is indeed tightly regulated, but not by way of changes in blood flow at the whole organ level. Thus, the tendency for tissue PO_2 to rise with RSR-13 could be counteracted by a decrease in number of capillaries being perfused without a change in overall organ blood flow, by more heterogeneity in RBC spacing or velocity in microvessels, by a relative shift of perfusion from supply vessels to arteriovenous shunts or by some combination of the above. Another possibility that could compensate for a potential rise in tissue PO_2 would be diffusive shunting, that is, increased diffusive flux of O_2 from small arteries and arterioles to adjacent veins and venules as a result of the higher PO_2 in such vessels. Additional experiments examining changes before and after RSR-13 infusion at the microvascular level will be required to examine these possibilities.

Acknowledgments

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Table 1. Blood gases, temperature and hematocrit.

	RSR-13		Control	
	Before	After	Before	After
PO_2, Torr	224 ± 34	238 ± 31[¶]	228 ± 29	234 ± 31
PCO2, Torr	39 ± 4	36 ± 3*	39 ± 9	36 ± 3
pH	7.42 ± 0.04	7.41 ± 0.03	7.43 ± 0.08	7.43 ± 0.04
P_{50}, Torr	35.4 ± 5.4	46.3 ± 4.1[§]	36.4 ± 4.2	36.4 ± 3.0
Hct, %	45 ± 2	44 ± 2[¶]	43 ± 2	42 ± 2[§]
Temp, °C	39 ± 0.4	38 ± 0.5[¶]	39 ± 0.6	39 ± 0.3

Tabled values are mean ± SD; n = 13 for each group. *$P < 0.05$; [¶]$P < 0.01$; [§]$P < 0.001$.

Table 2. Hemodynamic parameters.

	RSR-13		Control	
	Before	After	Before	After
Mean arterial pressure, mm Hg	73 ± 8	71 ± 8	73 ± 6	69 ± 10
Systolic pressure, mm Hg	173 ± 17	164 ± 15*	174 ± 12	174 ± 15
Diastolic, mm Hg	-4 ± 9	-2 ± 6	-5 ± 5	-5 ± 7
Heart rate, min^{-1}	452 ± 52	410 ± 50*	487 ± 44	470 ± 40*
Respiratory rate, min^{-1}	91 ± 12	89 ± 12	103 ± 14	97 ± 12[†]
Cardiac output, ml • min • kg	326 ± 34	249 ± 28[§]	360 ± 70	280 ± 38[§]
Periph vasc resist, mm Hg • ml^{-1} • min^{-1} • kg^{-1}	0.23 ± 0.03	0.29 ± 0.04[§]	0.21 ± 0.03	0.25 ± 0.03[§]

Tabled values are mean ± SD; n = 13 for each group. *$P < 0.05$; [†]$P < 0.01$; [§]$P < 0.001$

Table 3. Absolute blood flow in RSR-13 and control rats.

	RSR-13			Control		
Flow, ml • min^{-1} • g^{-1}	Before	After	% Change	Before	After	% Change
Heart	4.38 ± 1.30	4.51 ± 1.66	3	4.90 ± 1.61	4.87 ± 1.41	1
Brain	1.22 ± 0.24	1.19 ± 0.29	2	1.28 ± 0.19	1.27 ± 0.24	1
Kidney	5.63 ± 1.35	5.14 ± 0.67	9	5.26 ± 1.15	4.84 ± 0.76	8
Stomach	0.86 ± 0.28	0.55 ± 0.12[1]	37	0.82 ± 0.27	0.63 ± 0.13*	24
Intestine	2.03 ± 0.35	1.83 ± 0.44*	10	1.96 ± 0.33	1.84 ± 0.35	6
Liver, arterial	0.06 ± 0.03	0.09 ± 0.10	50	0.08 ± 0.07	0.12 ± 0.15	48
Liver, portal	4.60 ± 0.54	3.95 ± 0.89*	14	4.38 ± 0.81	3.72 ± 0.79*	15
Spleen	1.71 ± 0.36	1.57 ± 0.56	8	1.60 ± 0.60	1.25 ± 0.48*	22
Lungs	1.50 ± 1.22	1.98 ± 2.05	32	2.50 ± 2.25	1.65 ± 1.58	34
Diaphragm	0.51 ± 0.15	0.36 ± 0.10[5]	29	0.67 ± 0.22	0.52 ± 0.18*	22
Back/leg muscle	0.14 ± 0.05	0.08 ± 0.02[5]	43	0.14 ± 0.06	0.09 ± 0.02[1]	35
Carcass, ml • min^{-1} • g^{-1}	88.37 ± 11.91	63.97 ± 10.12[5]	28	90.26 ± 15.55	68.29 ± 9.68[5]	24

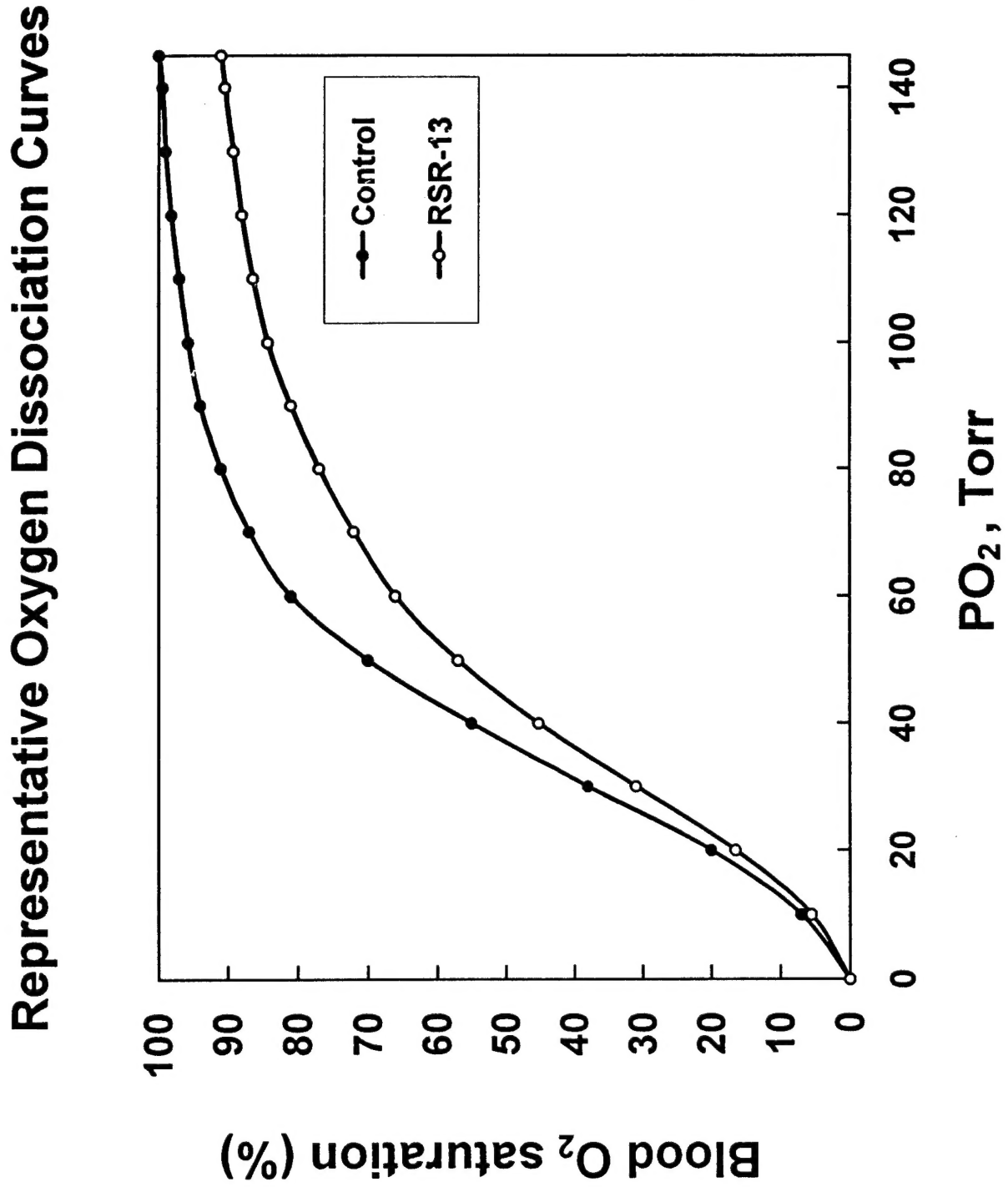
Tabled values are given ml • min^{-1} • g^{-1} (mean ± SD); n = 13 for each group. *$P < 0.05$; [1]$P < 0.01$; [5]$P < 0.001$

Table 4. Percent of cardiac output in RSR-13 and control rats.

	RSR-13			Control		
Flow, % cardiac output	Before	After	% Change	Before	After	% Change
Heart	4.20 ± 1.04	5.84 ± 2.42*	39	4.62 ± 1.54	5.95 ± 2.08*	29
Brain	1.92 ± 0.34	2.53 ± 0.83[1]	31	1.95 ± 0.48	2.41 ± 0.41 [1]	24
Kidney	12.86 ± 2.27	15.55 ± 2.17[1]	21	11.54 ± 1.98	13.66 ± 1.91[1]	18
Stomach	1.19 ± 0.31	1.00 ± 0.22	16	1.10 ± 0.38	1.07 ± 0.25	3
Intestine	13.37 ± 2.72	15.79 ± 3.98*	18	13.38 ± 3.06	15.86 ± 3.45*	19
Liver, art.	0.69 ± 0.29	1.38 ± 1.40	99	0.67 ± 0.37	1.55 ± 2.57	132
Liver, portal	15.53 ± 3.02	17.92 ± 4.07*	15	15.36 ± 3.43	17.79 ± 3.67	16
Spleen	0.97 ± 0.24	1.14 ± 0.31	17	0.88 ± 0.33	0.85 ± 0.28	3
Lungs	2.51 ± 2.01	4.17 ± 4.28*	66	3.47 ± 2.78	3.23 ± 3.39	7
Diaphragm	0.46 ± 0.17	0.42 ± 0.12	9	0.49 ± 0.13	0.52 ± 0.27	6
Carcass	61.83 ± 4.66	52.19 ± 6.92[5]	16	61.90 ± 6.14	54.89 ± 6.01[1]	11

Tabled values are given as percent of cardiac output (mean ± SD); n = 13 for each group. *$P < 0.05$; [1]$P < 0.01$; [5]$P < 0.001$

Figure 1



Legend to Figure 1

Representative blood O_2 dissociation curve of rat blood before and after infusion of RSR-13.

CONCLUSION

These studies show that the blood oxygen dissociation curve plays a major role in oxygen delivery, at least in the isometrically working isolated rabbit heart model (section I). Noteworthy was the finding that a rightward shift was able to produce partial reversal of ischemic effects during low flow ischemia. Whether this finding will prove true generally will require studies in other models and ultimately in man. The importance of this to military planners is that it has major potential implications for treatment of casualties. We believe this should be the subject of additional focused study.

A major surprise was the finding that a similar right shift of the oxygen dissociation curve, this time effected by infusion of the new drug RSR-13 in rats, did not produce evidence of altered blood flow to the body in general or to any organ studied (section III). This contrasts with earlier work from our laboratory, which showed that a left shift did evoke compensatory changes in blood flow to certain organs. Possible explanations include differing physiologic responses to left and right shifts in the range studied, compensatory changes at the microvascular level or confounding effects related to this drug per se.

These studies critically compared, for the first time, oxygen delivery by a purified hemoglobin solution versus red cells at equal hemoglobin concentration, flow rate and P_{50} (section II). We found that the two performed remarkably alike, with no evidence of either an increase in O_2 delivery due to the several postulated advantages of hemoglobin or a decrease due to vasoconstriction. We also found no change in vascular resistance in this model, though this could well have been due to approximately equal and opposite effects on resistance of reduced viscosity and decreased vessel caliber.

A more detailed discussion is found following the results section in each of the above sections.